Identification and characterization of glycoside hydrolase family 32 enzymes from Aspergillus niger

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CHAPTER 4

Exo-inulinase of A*spergillus niger* n402: a hydrolytic enzyme with significant transfructosylating activity

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Abstract

The exo-inulinase gene *inuE* of *Aspergillus niger* N402 was cloned and heterologously expressed in *Escherichia coli*. The purified enzyme (AngInuE) was able to hydrolyse sucrose, raffinose, inulin and levan, displaying a sucrose:inulin (S/I) hydrolysis ratio of approximately 2.3, which is characteristic for a typical exo-inulinase. Apart from hydrolysis, the enzyme had a significant transfructosylating activity with increasing sucrose concentrations. The oligosaccharides produced included 1-kestose, nystose, pentakestose, and low molecular weight inulins of the neo-series type. The molecular mass of the *E. coli* produced and purified AngInuE protein, lacking the signal sequence peptide, was approx. 57 kDa, close to the calculated molecular mass of the mature protein. This AngInuE enzyme thus was active in its monomeric, non-glycosylated state. Contradicting data on the hydrolysis versus transfructosylation activity have been published on the identical sucrose: sucrose 1-fructosyltransferase (1-SST, a dimeric and glycosylated) enzyme of *Aspergillus foetidus*, and the almost identical (monomeric but glycosylated) exo-inulinases (InuE and Inu1) of *A. niger* 12 and *Aspergillus awamori*. Our data clearly show that the *A. niger* N402 InuE enzyme is a broad specificity exo-inulinase that also has significant transfructosylating activity with sucrose. Site-directed mutants in the Glycoside Hydrolase family 32 conserved domain G of AngInuE displayed strongly reduced hydrolytic activities on sucrose, inulin and levan. Apparently, the domain G amino acid residue Ser469 is important for catalytic efficiency, with a clear role in hydrolysis of both sucrose and fructans.

1. Introduction

Fructans are composed of multiple fructose units linked primarily by β-2,1- (inulins) or β-2,6-glycosidic bonds (levans). Many plants such as Jerusalem artichoke, chicory and dahlia make inulin type fructans and store these in their roots (Cairns, 2003). Inulins are used by the food industry to produce fructose-rich syrups, as fat substitutes or for the production of short chain oligosaccharides that are used as a prebiotic (for reviews see Vijn & Smeekens, 1999; Ritsema & Smeekens, 2003a; Ritsema & Smeekens, 2003b). Fructans are synthesized and hydrolyzed by the action of enzymes belonging to the glycoside hydrolase families 32 (GH32, in plants and fungi) and GH68 (in bacteria) (http://www.cazy.org; Coutinho & Henrissat, 1999). Hydrolysis of fructans is performed by endo-inulinase (EC 3.2.1.7) or exo-inulinase (EC 3.2.1.80) enzymes (Vijn & Smeekens, 1999). In the filamentous fungus *Aspergillus*, both types of inulinases have been identified, showing that this genus is well equipped to utilize fructans from plant material (Ettalibi and Baratti, 1987; Ohta *et al*., 1998; Arand *et al*., 2002; Moriyama *et al*., 2004).

Over the years, a number of transfructosylating enzymes as well as exo-inulinases from *Aspergillus* species have been described that all share a near identical amino acid sequence (Rehm *et al*., 1998; Arand *et al*., 2002; Moriyama *et al*., 2004). Recently, we reported the identification and transcriptional analysis of fructan modifying enzymes identified in the genome of *A. niger* (Yuan *et al*., 2006; Pel *et al*., 2007). *In silico* and expression analysis indicated the presence of single endo-inulinase (InuA; EC 3.2.1.7) and exo-inulinase (InuE; EC 3.2.1.80) enzymes in *A. niger*. Comparison of the deduced amino acid sequence of the putative *A. niger* exo-inulinase InuE (from here onward referred to as AngInuE) with other fungal exo-inulinases and transfructosylating enzymes showed that AngInuE is identical to the *A. foetidus* 1-SST. Surprisingly, the latter enzyme has been classified as a fructosyltransferase active on sucrose without any inulin or levan hydrolysing activity (Rehm *et al*., 1998). Moreover, AngInuE differs in only three amino acids from the exo-inulinase (InuE) of *A. niger* 12 (Moriyama *et al*., 2003), an enzyme that reportedly hydrolyzes inulin but not levan. AngInuE also displays a high sequence identity (91%) with the inulin- and levan hydrolysing exo-inulinase Inu1 of *A. awamori* (Arand *et al*., 2002). Both the *A. niger* 12 InuE and the *A. awamori* Inu1

have been characterized as exo-inulinases lacking any detectable transfructosylation activity on sucrose (Arand *et al*., 2002; Kulminskaya *et al*., 2003; Moriyama *et al*., 2003). In this communication we demonstrate that the *A. niger* N402 AngInuE is actually a true exo-inulinase with significant transfructosylation activity on sucrose

In the secondary structure of family GH32 proteins, eight well-conserved domains (designated A, B, B1, C, D, E, F and G, respectively) can be distinguished (Ohta *et al*., 1998; Pons *et al*., 1998). Three of these domains (A, D and E) contain highly conserved acidic residues that are located in the active site of members of family GH32. Apart from the well described "sucrose binding box" (domain A), which plays an important role in catalysis, substrate- and product diversity (Ritsema *et al*., 2004; Ritsema *et al*., 2005), little is known regarding the contributions of the other conserved domains. Domain G is located in the cleft between the 5-bladed β-propeller and β-sandwich structures in family GH32 proteins, clearly separate from the active site. Here we report analysis of site-directed mutants in domain G of AngInuE, demonstrating that domain G in fact plays a profound role in catalysis.

2. Materials and Methods

2.1 Strains and media

A. niger strain N402 used in this study was derived from the wild-type strain *A. niger* van Tieghem (CBS 120.49, ATCC 9029; Bos *et al*., 1988). The strain was grown in Minimal Medium (MM) (Bennet & Lasure, 1991) containing 7 mM KCl, 11 mM KH_2PO4, 70 mM $NaNO_3$; 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 178 nM H_3BO_3, 25 nM $MnCl_2$, 18 nM $FeSO_4$, 7.1 nM, $CoCl_2$, 6.4 nM $CuSO_4$, 6.2 nM Na_2MoO_4 and 174 nM EDTA. *Escherichia coli* strains TOP 10 and BL21 (DE3) STAR were used for general cloning and for heterologous protein expression, respectively (Invitrogen).

2.2 Amplification and cloning of *inuE*

A cDNA library of *A. niger* N402 was created from total RNA of fungal mycelia grown in minimal medium containing inulin as sole carbon source (Yuan & Ram, unpublished results). The cDNA library was used as template for amplification of

inuE (accession number DQ233222) using the forward primer *inuEGateF*, which excludes the fragment encoding the N-terminal signal sequence (the first 19 amino acids), and the reverse primer *inuEGateR* (Table 1). The construct was inserted into the *E. coli* Gateway vector pDEST17, enabling the addition of an N-terminal 6x histidine affinity tag (Invitrogen). *Pwo* polymerase (Roche) was used for amplification. PCR cycling conditions used were: initial denaturation for 2 min at 94°C, 30 cycles of 15 sec denaturation at 94°C, annealing at 55°C for 30 sec and elongation at 72°C for 90 sec, followed by a final elongation step of 7 min at 72°C. PCR products were purified using the Geneclean II kit (Q.Biogene), followed by inserting into the Gateway-compatible vectors (Invitrogen). Gateway cloning of *inuE* was performed as described by the manufacturer. In short, the purified amplicon was inserted by site specific recombination into the entry vector pDONR201, followed by subsequent transfer to the expression vector pDEST17. The integrity of the insert was confirmed by DNA sequencing (ServiceXS).

2.3 Construction of AngInuE mutant proteins

To create a triple mutant of AngInuE that is identical to InuE of *A. niger* 12 described by Moriyama *et al.* (2004), point mutations were successively introduced by inverse PCR using 30-40 bp complimentary primers and *Taq* Expand long template polymerase (Roche). Three consecutive sets of primers were used to introduce the mutations Gln199His (primer pair *0185Q199Hfw* and *0185Q199Hrev*), Ser476Gly (primer pair *0185S476Gfw* and *0185S476Grev*) and Ser499Thr (primer pair *0185S499Tfw* and *0185S499Trev*) (Table 1).

To determine the importance of the putative fructan binding domain G (SVEVF motif) on AngInuE activity, mutations were introduced as described above by replacement of the conserved serine residue (Ser469Thr and Ser469Val, primers depicted in Table 1). Amplification conditions were as recommended by the manufacturer. Following PCR, samples were treated with *DpnI* to hydrolyse wild-type methylated pDEST17-*AnginuE* (plasmid DNA from *E. coli* TOP 10), and subsequently transformed into *E. coli* TOP 10 and BL21 (DE3) STAR. The integrity of the mutants was confirmed by DNA sequencing (ServiceXS).

Table 1. Primers used in this study. Changed nucleotides are shown underlined

Name	Sequence
inuEGateF	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTTCAACTATGACCAGCCTTACC
inuEGateR	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATTCCACGTCGAAGTAA
0185Q199Hfw	GACGAGTCCCATAAATGG
0185Q199Hrev	CCATTTATGGGACTCGTC
0185S476Gfw	GTGCCGGATAGCACTGGCATGGTGAGGTTGAG
0185S476Grev	CTCAACCTCACCATGCCAGTGCTATCCGGCAC
0185S499Tfw	GGAGGCCAAGGTGAGACGACTTTGACGGCTCAGATC
0185S499Trev	GATCTGAGCCGTCAAAGTCGTCTCACCTTGGCCTCC
InuES469T forw	GTATCTTCGTCGACAGGTCCACCGTCGAGGTATTCGGAGG
InuES469T rev	CCTCCGAATACCTCGACGGTGGACCTGTCGACGAAGATAC
InuES469V forw	GTATCTTCGTCGACAGGTCCGTCGTCGAGGTATTCGGAGG
InuES469T forw	CCTCCGAATACCTCGACGACGGACCTGTCGACGAAGATAC

2.4 Protein expression and purification

Starting cultures of *E. coli* BL21 STAR (Invitrogen) containing the respective expression vectors were grown at 37°C for 16 h. Subsequently, 10 ml of each culture was used to inoculate 1 litre fresh Luria Bertani medium, followed by growth overnight at 18°C with agitation. To increase expression of soluble protein, cells were heat shocked at 50°C for 10 min with agitation, followed by induction using isopropyl-ß-D-thiogalactopyranoside (IPTG, 1 mM final) and further growth for 6 h at 18°C (until OD_{600nm} 0.8-1.0). Cells were harvested by centrifugation (10 min, 4°C, 4000 x *g*) and cell pellets were resuspended in 10 ml 50 mM sodium phosphate buffer, pH 8, containing 250 mM NaCl, 10 mM imidazole and 5 mM of β-mercaptoethanol. Cell lysis was done by sonification on ice (7 cycles of 15 sec at 8

micron with 30 sec intervals). Cell free lysate was obtained by centrifugation (20 min at 4°C, 10,000 x *g*). Active protein was purified from the cell-free lysates using Ni-NTA affinity chromatography (Sigma-Aldrich) and concentrated using a 30 kDa ultracentrifugation filter (Microsep). The final concentrated volume (500 µl) was loaded on a Superdex 200 HR gel filtration column (Sigma-Aldrich) and fractionated using a 20 mM Tris-HCl buffer pH 7.4 containing 200 mM NaCl at a flow speed of 0.3 ml per min. Active eluted fractions (0.6 ml each) were pooled, followed by exchanging the buffer to 50 mM acetate, pH 5.0 (PD10 desalting column, Amersham Biosciences) and concentration (30 kDa ultracentrifugation filter, Microsep). Protein concentration was determined using the Bradford reagent (Bio-Rad). Enzyme activity was determined after all purification steps by incubation with 100 mM sucrose in 50 mM acetate buffer, pH 5, at 37°C, and determining the rate of initial glucose and fructose release as described below. Purification of the protein was verified by analysis of the fractions by 10% SDS-PAGE and Coomassie staining.

Protein size estimation was performed by blue native page electrophoresis (Nijtmans *et al.*, 2002), size exclusion chromatography (Superdex 200 HR, Amersham) as well as by mass spectrometry (MALDI-TOF MS). For MALDI-TOF MS analysis, 0.75 µl of protein (0.4 mg ml^{-1} in 20 mM NaCl) was spotted and mixed on a MALDI target with an equal volume of matrix (10 mg ml^{-1} sinapinic acid in a 50:50:0.1 (v/v/v) water: acetonitrile: trifluoroacetic acid solution). Spectra were acquired in positive linear mode on a Voyager DE-Pro mass spectrometer (Applied Biosystems) and calibrated using bovine serum albumin as mass standard.

2.5 Activity assays

Enzyme activity was quantified spectrophotometrically by separate measurements of the released glucose and fructose using the D-glucose/D-fructose kit (Roche). Transfructosylation was measured by calculating the difference between released glucose and fructose. Optimal pH was determined by measuring enzymatic activity (initial rates) at 37^{o}C in 50 mM phosphate-citrate buffer containing 100 mM sucrose, with a pH range of 4 to 7, using 0.5 pH unit increments. Optimal temperature was determined by measuring enzyme activity using 100 mM sucrose in 100 mM acetate buffer at pH 4, with a temperature range of 37 to 70°C. To avoid substrate

autohydrolysis, the effect of substrate concentration on enzyme activity was determined under suboptimal conditions, in 100 mM acetate buffer at pH 5.0, 37^{o}C. Enzyme activity was measured in triplicate using 64 ng of purified enzyme and a sucrose range of 15 concentrations (2 mM to 1 M). The sucrose to inulin (S/I) hydrolysis ratio was determined by adding 64 ng of purified enzyme (in 20 µl) to 200 µl of sucrose or inulin (pH 5.0, 37^{o}C), at concentrations ranging from 8.7 to 43.4 g l^{-1}. The molecular mass of the inulin (Frutavit TEX, Sensus) was estimated at 4341.8 g mol^{-1}, based on an average determined chain length of 24.1 fructose units. All activity data is based on triplicate activity measurements in 1 minute intervals during 8 to 12 min of incubation.

2.6 Substrate specificity and product range

To analyse substrate specificity and the product profiles of AngInuE (and mutants derived), 55 ng of purified enzyme was mixed with 200 µl of the appropriate substrate (at 100 mM; pH 5.0) and incubated between 10 min and 7 days. Substrates used include sucrose (Sigma-Aldrich), 1-kestose (Fluka), raffinose (Sigma-Aldrich), nystose (Fluka), inulin (Frutavit TEX, Sensus) and levan (from *Bacillus subtilis*, kind gift from Dr. H. Raaijmakers, Royal Cosun). Products were characterized by thin layer chromatography (TLC) as well as by high performance anionic exchange chromatography (HPAEC; Dionex Corporation), as described before (Ozimek *et al*., 2006; Goosen *et al*., 2007). Pure standards of glucose, fructose, sucrose, 1-kestose, 6-kestose, nystose, pentakestose and the neo-series inulins neokestose (3b), 4c and 4b (Shiomi *et al.*, 2005) were used to calibrate the HPAEC column elution times.

3 Results

3.1 Cloning, heterologous expression and purification of AngInuE

To obtain the full length open reading frame of *inuE*, PCR amplification was performed on a cDNA library of *A. niger* N402 grown on inulin, yielding a single product of the expected size (1611 bp). The *inuE* amplification product was inserted into the Gateway donor vector (pDONR 201), followed by sub-cloning into the

destination vector pDEST17. The presence of a single intron in *inuE* at nucleotide positions 394 to 453 was confirmed when comparing the nucleotide sequence of the cDNA clone with that of *inuE* from the genomic sequence (accession number DQ233222). No activity was detected in *E. coli* extracts when a standard induction temperature of 37^{o}C was used. The amount of soluble AngInuE protein in *E. coli* was successfully increased by heat shock in combination with low temperature induction (Strandberg & Enfors, 1991). Affinity purified AngInuE protein displayed a size of approximately 89 kDa, judging from denaturing- and blue-native PAGE. Size exclusion chromatography resulted in the elution of active protein with a molecular mass of approximately 50 kDa. The same sample was analysed by mass spectrometry (MALDI-TOF MS), which confirmed the expected molecular mass (57 kDa) of monomeric AngInuE.

3.2 Activity of AngInuE

The effect of pH and temperature changes on AngInuE activity was determined using sucrose and inulin as substrates. Maximal rates of hydrolysis of both sucrose and inulin were observed at a temperature of 60^{o}C and at a pH lower than 4. At this condition significant abiotic hydrolysis of the substrate occurred as well, making a reliable determination of the enzyme's activity difficult. For this reason, all further analysis were performed at pH 5 and 37^{o}C, with no detectable abiotic hydrolysis of the substrates; at these conditions the enzyme displayed approximately 60% of its optimum activity.

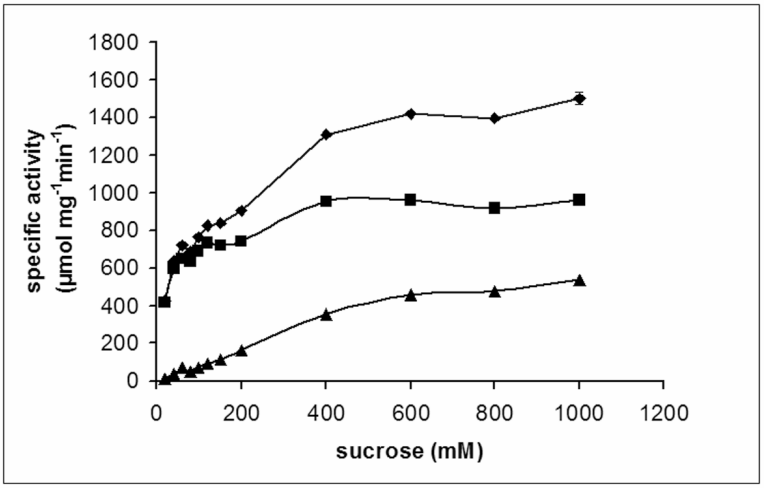
Incubating AngInuE with increasing concentrations of sucrose produced a typical Michaelis-Menten activity graph, where a *Km* of 31.7 (± 4.8) mM and *Vmax* of 973.2 (± 30.7) μmole mg^{-1} min^{-1} were determined for hydrolysis (Fig. 1 A). Transfructosylation activity increased with increasing sucrose concentrations (*Vmax* of 657.1 (± 62.2) μmole mg^{-1} min^{-1}, *Km* of 344 (± 57.5) mM), yielding various short chain oligosaccharides (Fig. 1 B). At a sucrose concentration of 20 mM, InuE displayed a transfructosylation activity of approximately 3% (12.5 μmole mg^{-1} min^{-1}) of total enzyme activity (430.3 μmole mg^{-1} min^{-1}). Increasing the sucrose concentration to 1 M brought about a significant increase in transfructosylation activity to approximately 36% (539.5 μmole mg^{-1} min^{-1}) of total enzyme activity

(1501.3 µmole mg^{-1} min^{-1}). The catalytic rates (*kcat*) for AngInuE sucrose hydrolysis and transfructosylation were determined as 928.9 (± 30.7) s^{-1} and 627.3 (±62.2) s^{-1}, respectively, based on a molecular mass of the native mature monomeric protein (calculated as 57.27 kDa). When measuring inulin hydrolysis, we noticed that AngInuE was not saturated by inulin up to a concentration of 10 mM, reaching a specific activity of 402.9 µmol mg^{-1} min^{-1}. Above this concentration the inulin was less soluble, making accurate determinations of kinetic parameters impossible. The ratio of the initial rates of hydrolysis of sucrose and inulin (S/I ratio) was calculated to be 2.3 (± 0.4) at the most.

3.3 Substrate specificity and product range

AngInuE was incubated with various substrates to determine its substrate specificity, as well as the range of products obtained. The enzyme was incubated with 100 mM of 1-kestose or nystose, alone or in combination with 100 mM of sucrose, and with 100 mM raffinose, or 1% (w/v) of inulin or levan, respectively. TLC analysis showed that InuE partially hydrolysed levan and produced fructose. A similar amount of inulin was hydrolysed completely to fructose in the same amount of time (Fig. 2). HPAEC analysis showed that AngInuE incubated from 10 minutes to overnight with 100 mM of sucrose produced free fructose, glucose and minor amounts of short oligosaccharides. When increasing the sucrose concentration to 1 M, larger amounts of 1-kestose, 6-kestose and neokestose were found (overnight incubations shown in Fig. 3 A). With 100 mM of 1-kestose as substrate, a similar product profile was observed, except for the presence of small amounts of nystose and most likely the neoseries inulin 4c (Fig. 3 B). Nystose gave a similar product profile as that found for sucrose and 1-kestose, but also a small amount of pentakestose (GF4) was formed (Fig. 3 C). Addition of 100 mM of sucrose to either 1-kestose or nystose did not alter the product profile, but led to an overall increase in concentration of all products. Raffinose was also hydrolysed, releasing free fructose and melibiose (α-D-galactose-(1,6)-α-D-glucose) (data not shown).

(A)



(B)

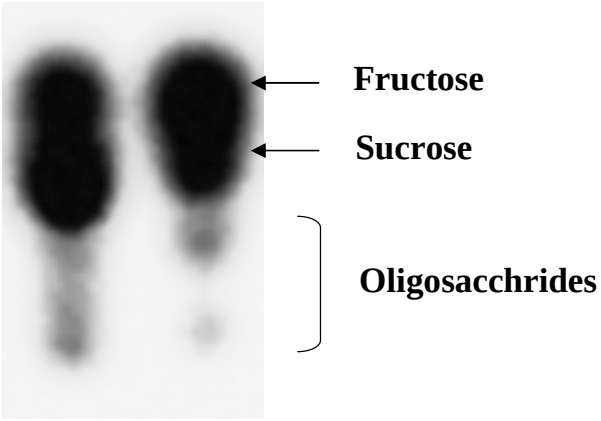
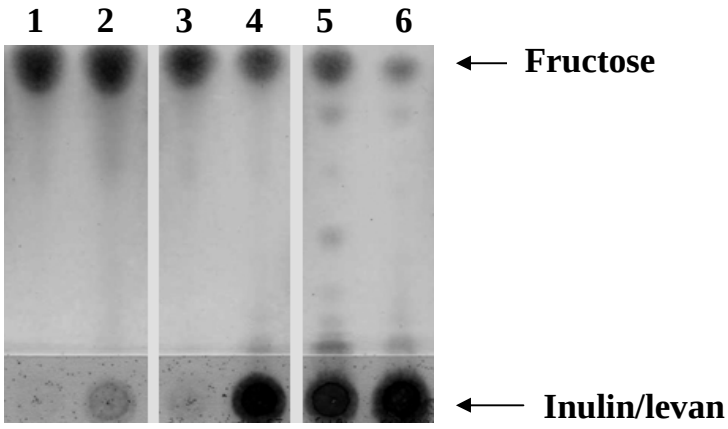


Figure 1. (A) Effects of sucrose concentration on AngInuE activity. Activity was determined by measuring the amount of glucose and fructose released from the initial reaction of InuE (64 ng) incubated with 11 sucrose concentrations ranging from 20 mM to 1 M in 50 mM acetate buffer pH 5.0 at 37°C. Total- (♦), hydrolysis- (■) and transfructosylation (▲) activities are depicted. (B) TLC plate showing free fructose, sucrose and transfructosylation products after 2 (lane 1) and 5 (lane 2) days of incubation of 64 ng AngInuE with 1 M of sucrose, pH 5.0, 37^{o}C. Products identified are indicated.

(A)



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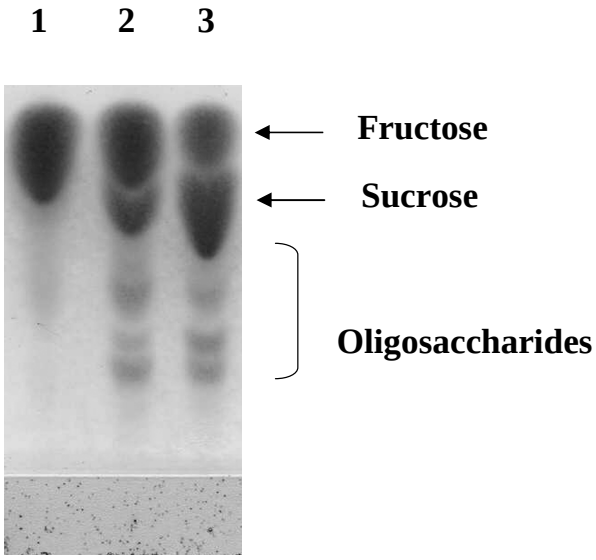
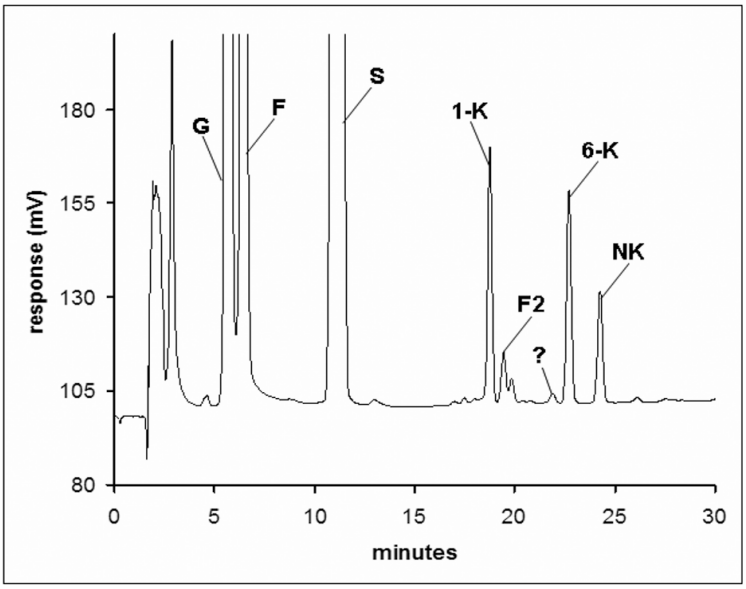


Figure 2 (A) TLC analysis wild-type AngInuE (lanes 1 and 2), AngInuE Ser469Thr (lanes 3 and 4) and AngInuE Ser469Val (lanes 5 and 6) (6 µg each) incubated for 7 days (37^{o}C, pH 5.0) with 200 µl of 1% (w/v) of inulin or levan (odd and even numbered lanes respectively). Inulin, levan and released fructose (F) are indicated. (B) TLC analysis of products synthesized by wild-type AngInuE (lane 1), AngInuE Ser469Thr (lane 2) and AngInuE Ser469Val (lane 3) (1 µg each) in the presence of 1M sucrose (37^{o}C, pH 5.0) for 7 days.

(A)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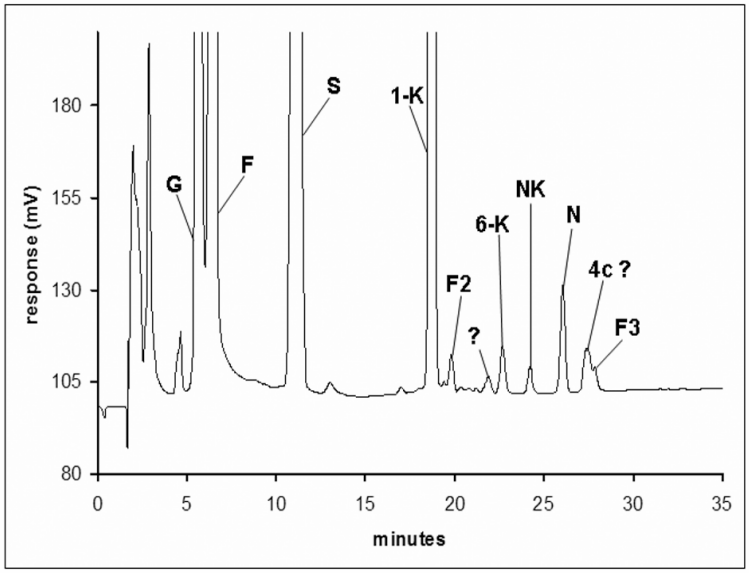
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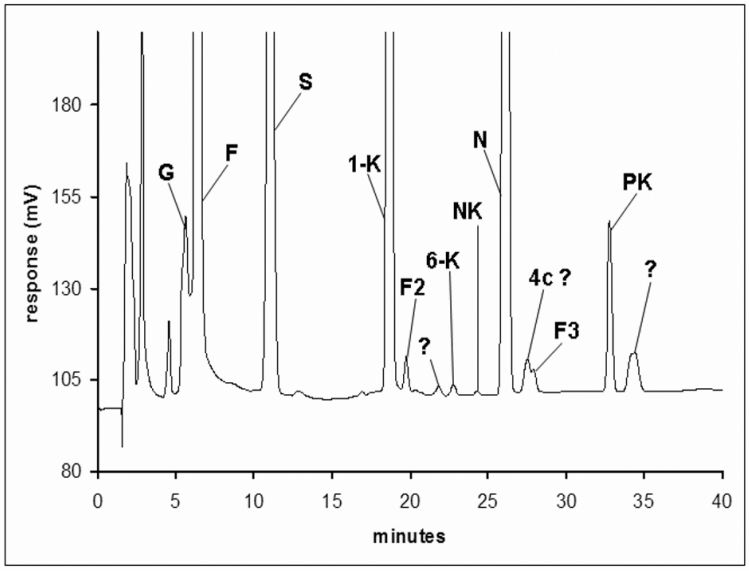

Figure 3 HPAEC chromatogram depicting AngInuE product profiles; 55 ng of enzyme was incubated (37°C, pH 5.0, overnight) with 1 M sucrose (A), 100 mM 1-kestose (B) and 100 mM nystose (C). G, glucose; F, fructose; S, sucrose; 1-K, 1-kestose; F2, difructose; 6-K, 6-kestose; NK, neokestose; N, nystose; 4c, neo-series inulin 4c (see text); F3, trifructose; PK, pentakestose. Unknown products or products with unclear identity are indicated by a question mark.

3.4 Characteristics of AngInuE mutants

The exo-inulinase of *A. niger* 12 (InuE, Moriyama et al. 2003), which is able to hydrolyze inulin but not levan, and which lacks any detectable transfructosylation activity, differs in only three amino acids from AngInuE. To determine the possible effect of these three amino acid differences, all three amino acids of AngInuE were changed into their InuE counterparts (Gln199His, Ser476Gly and Ser499Thr). AngInuE and the triple mutant displayed comparable substrate specificity and product profiles (data not shown). Also mutations in the family GH32 putative fructan binding motif (SVEVF; domain G; Pons et al. 2002; Yuan et al. 2006) were made. Indeed, in mutants Ser469Thr and Ser469Val levan hydrolysis was weak or absent (Fig. 2 A,

lanes 4 and 6), whereas hydrolysis of 10 mM inulin was reduced to 64.8 and 38.9 µmol mg^{-1} min^{-1}, respectively (approximately 16 and 10 % of wild type specific activity; see Fig. 2 A, lanes 3 and 5). Surprisingly, the Ser469Thr and Ser469Val mutations also caused a reduction in the *kcat* for sucrose hydrolysis, to 64.9 (± 1.6) and 30.5 (± 0.3) s^{-1}, respectively (approximately 7 and 3 % of wild type maximum velocity). Interestingly, the affinities of the Ser469Thr and Ser469Val mutant proteins for the hydrolysis of sucrose increased, from a *Km* of 31.7 (± 4.8) mM for wild type, to *Km* values of 8.5 (± 1.7) and 16 (± 0.8) mM, respectively. Mutagenesis of Ser469 also influenced transfructosylation activity of AngInuE with sucrose (Fig 2 B). At sucrose concentrations of 20 mM to 1 M, mutant Ser469Thr displayed transfructosylation activities of approximately 30 to 50% of total enzyme activity, respectively. The Ser469Val mutant displayed transfructosylation values between 14 to 25% of total enzyme activity. The transfructosylation/hydrolysis ratio in mutant Ser469Thr, but not in Ser469Val, is thus clearly higher than in wild type AngInuE.

4 Discussion

This paper deals with the cloning, heterologous expression and biochemical characterization of the single exo-inulinase (here referred to as AngInuE) identified in *A. niger* CBS 513.88 and N402 (Yuan *et al.*, 2006). Biochemical characteristics of the purified AngInuE protein that was overproduced in *E. coli* were compared to those of the orthologous enzymes of *A. foetidus* (1-SST, 100% identity, Rehm *et al.*, 1998), *A. niger* 12 (InuE, 99% identity, Moriyama *et al.*, 2004) and *A. awamori* (Inu1, 91% identity, Arand *et al.*, 2003).

AngInuE expressed in *E. coli* was active as a non-glycosylated monomeric enzyme of 57 kDa which displayed primarily hydrolytic activity on sucrose, inulin and levan. Second to that, transfructosylation activity was also detected with sucrose and the small oligomeric inulins 1-kestose and nystose. Transfructosylation products included 1-kestose, 6-kestose, pentakestose and the neo-series inulins (Fig. 3). Increasing the substrate concentration resulted in an increase in transfructosylation activity (Fig. 1 A). Furthermore, AngInuE displayed a S/I ratio of 2.3 (± 0.4) indicating that it is a true exo-inulinase. This value correlates well with the data of

Moriyama et al. (2003) showing that the InuE of *A. niger* 12 has an S/I ratio of 4.3. Inulinases typically display an S/I ratio in the range of 0.5 to 18.5, whereas true invertases display S/I values of several thousands (Vandamme & Derycke, 1983).

In contrast to AngInuE, the 100% identical protein 1-SST from *A. foetidus* was inactive towards inulin, its major activity being transfructosylation of sucrose (up to 70% at 1 M sucrose, compared to 36% for AngInuE) (Rehm *et* al., 1998; this study). On the contrary, no transfructosylation activity was observed for the closely related Inu1 and InuE enzymes when incubated at sucrose concentrations ranging from 150 - 200 mM (Kulminskaya *et al*., 2003; Moriyama *et al*., 2003).

Exo-inulinases generally possess the ability to hydrolyze both β-2,1- (sucrose, inulin) as well as β-2,6- (levan) glycosidic linkages, releasing free fructose in the process (http://www.expasy.ch/enzyme/; Bairoch, 2000). AngInuE clearly released fructose from both inulin and levan, although at a lower rate from levan. Similar results have been found for the Inu1 exo-inulinase of *A. awamori* (Arand *et al.*, 2002; Kulminskaya *et al.*, 2003). But these results differ clearly from those found with the 1-SST of *A. foetidus* (Rehm *et al.*, 1998) and the InuE of *A. niger* 12 (Moriyama *et al.*, 2004): for these enzymes no hydrolysis of levan could be detected. Comparison of the deduced amino acid sequences of AngInuE and the InuE of *A. niger* 12 showed that these two enzymes differ in only three amino acids. The three amino acids that differ are chemically similar, and are not present in one of the eight conserved motifs defined for family GH 32 (Ohta *et al.*, 1998; Pons *et al.*, 2002). From the three-dimensional structure of the *A. awamori* exo-inulinase (Nagem *et al.*, 2004) we observed that these changes are not in the vicinity of the catalytic core of the enzyme, and therefore are unlikely to have a direct effect on substrate utilization. No differences in activity or product specificity were observed with the AngInuE triple mutant, with the amino acid sequence changed into that of the *A. niger* 12 InuE, thus confirming that these three amino acids are not responsible for the differences in specificity found between the orthologous enzymes. Clearly, unnoticed differences in activity assay and/or protein production conditions may cause these differences in enzyme properties. It also remains possible that the observed differences between AngInuE, 1-SST and InuE are caused by the absence of glycosylated amino acids in the *E.coli*-expressed AngInuE. Previous studies with other proteins have shown that non- and over- glycosylated forms of one and the same protein may have an effect on

protein kinetics, stability and even specificity (Shipley *et al.*, 1993; Wicker-Planquart *et al*., 1999; Barbier *et al*., 2000).

The molecular mass of AngInuE was determined using a number of independent techniques. Although the methods gave somewhat different values, they all indicate that the enzyme was active as a monomer of about 57 kDa. The size was in the same range as that of the deglycosylated Inu1 from *A. awamori* (69 kDa) (Arand *et al.*, 2002) and InuE from *A. niger* 12 (81 kDa) (Moriyama *et al.*, 2004). However, it clearly differs from the 180 kDa found for the 1-SST of *A. foetidus* (Rehm *et al.*, 1998) and the 210-240 kDa found for the SUC2 from *A. niger* N402 (Wallis *et al.*, 1997). The two latter enzymes are active as dimers. Substantial variation in molecular mass of exo-inulinases from other *Aspergillus* has also been reported: *A. versicolor* being 230 ± 20 kDa (Kochhar *et al.*, 1997), *A. candida* being 54 kDa (Kochhar *et al*., 1999) and *A. ficuum* being 74 kDa (Ettalibi *et al.*, 2001). Differences in size may be attributed at least partly to gel electrophoresis mobility shifts reflecting substantial glycosylation of the proteins produced in the fungal hosts. The possibility also exist that different isoforms of the enzyme were present and functionally active, as has been shown before for the intracellular and extracellular invertase of *S. cerevisiae* (Rubio & Maldonado, 1995; Straathof *et al.*, 1986) and the two different isoforms of the extracellular exo-inulinase of *A. fumigatus* (Gill *et al.*, 2006).

A structural feature widely present in family GH32 enzymes is the C-terminal β-sandwich domain, which contains the sequence motif SVEVF (GH32 domain G). Although the precise function of this domain is unknown, its presence appears to be essential for activity. Altenbach *et al*. (2005) showed that a chimera consisting of the N-terminal part of *Festuca* sucrose: sucrose 1-fructosyltransferase (1-SST) and the C-terminal part of the barley sucrose: fructan 6-fructsyltransferase (6-SFT) resulted in truncation of the C-terminal β-sandwich domain during heterologous expression in *Pichia pastoris*, resulting in a catalytically inactive protein. Furthermore, Kim *et al*. (2005) reported that amino acid substitutions in the N-terminal domain (NTD) of the family GH32 *Arthrobacter* sp. S37 endoinulinase (EnIA), or truncation thereof, caused reduction and even loss of enzyme activity. Although different in structure from the C-terminal β-sandwich domain, Kim *et al*. (2005) proposed that both these domains could be involved in protein dimerization and binding of carbohydrates. Within the β-sandwich domain, the sequence SVEVF (family GH32 domain G) is

highly conserved among fungal polymeric fructan hydrolysing enzymes, but not in invertases (Yuan *et al*., 2006), and has been proposed to play a role in polymer binding (Burne *et al.*, 1992; Ohta *et al.*, 1998; Moriyama *et al.*, 2003). Domain G is located in a cleft between the 5-bladed β-propeller and the β-sandwich domain. Also 3D structural analysis of the exo-inulinase of *A. awamori*, and the FEH protein of *Cichorium. intybus* indicated that the β-sandwich domain might be involved in fructan binding, based on the presence of glycerol molecules in the cleft situated between the two structural domains (Arand *et al*., 2003; Verhaest *et al*., 2005). In this study we have shown that substitution of the highly conserved Ser469 of domain G into a structurally and biochemically similar residue (Thr469) decreased hydrolytic activity of AngInuE on sucrose, inulin and levan. The importance of this residue in hydrolysis of sucrose and fructans was further supported by substitution of Ser469 into a hydrophobic residue (Val469), which almost completely abolished activity on sucrose, inulin and levan. The presence of the SVEVF motif in AngInuE, especially the conserved Ser469, thus is of crucial importance for overall catalytic efficiency. Further mutagenesis studies are needed to elucidate the precise function of this domain in AngInuE and in the family GH32.

To conclude, the biochemical characteristics and the substrate/product specificity of AngInuE from *A. niger* have been determined, and compared to those of closely related exo-inulinases/transfructosylation enzymes of *Aspergillus* species published earlier. We have shown that *A. niger* AngInuE is an exo-inulinase, hydrolysing sucrose and the fructans inulin and levan. At the same time it also displayed clear transfructosylation activity with sucrose, producing small oligomers of inulin and levan, as well as inulins of the neo-series type. Furthermore, mutagenesis in the conserved and putative family GH32 fructan binding domain G showed that this domain is important for catalytic efficiency of AngInuE, especially with regards to hydrolysis of sucrose and fructans. Minor changes in this domain may also influence the hydrolysis to transfructosylation activity of AngInuE, further stressing the importance of this domain in enzyme activity.

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